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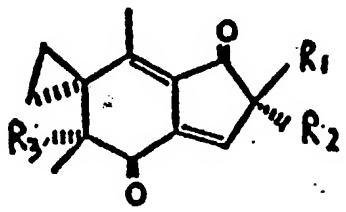

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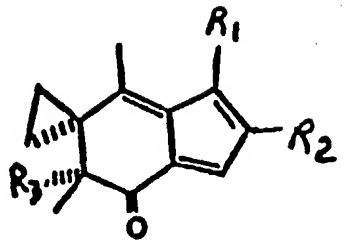

(II)

(57) Abstract

A method of inhibiting tumor cell growth in a subject is provided comprising contacting the tumor with a therapeutic amount of an illudin S or illudin M analog having structure (I) or (II), wherein the analog is capable of inhibiting tumor cell growth without excessive toxicity to the subject and wherein R_1 is an alkyl or hydrogen; R_2 is an alkyl; and R_3 is an alcohol or ester.

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ILLUDIN ANALOGS AS ANTI-TUMOR AGENTS

BACKGROUND OF THE INVENTION

Multiple agent chemotherapy has curative potential in some hematologic malignancies and advanced rapidly proliferating solid tumors. Curative chemotherapy has benefitted from the discovery of new, relatively non-cross resistant agents, and more effective use of existing agents. Interventions which increase the efficacy of conventional agents include more effective regimens for multiple drug administration, minimization of drug toxicities and increased use of adjuvant, surgical or radiation therapy.

Despite recent advances, patients with many types of malignancies remain at significant risk for relapse and mortality. After relapse, some patients can be reinduced into remission with their initial treatment regimen. However, higher doses of the initial chemotherapeutic agent or the use of additional agents are frequently required, indicating the development of at least partial drug resistance. Recent evidence indicates drug resistance can develop simultaneously to several agents, including ones to which the patient was not exposed. The development of multiple-drug resistant (mdr) tumors may be a function of tumor mass and constitutes a major cause of treatment failure. To overcome this drug resistance, high-dose chemotherapy with or without radiation and allogenic or autologous bone marrow transplantation is employed. The high-dose chemotherapy may employ the original drug(s) or be altered to include additional agents. The feasibility of this approach has been demonstrated for hematopoietic and solid tumors. The development of new drugs non-cross resistant with mdr phenotypes is required to further the curative potential of current regimens and to facilitate

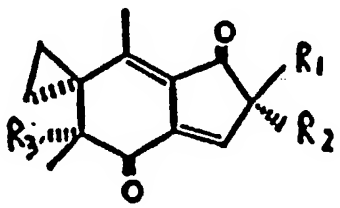
curative interventions in previously treated patients.

Recently, the in vitro anti-tumor activity of a novel class of natural products called illudins was examined in Kelner, M. et al., Cancer Res. 47:3186 (1987), incorporated herein by reference. Illudin S and M are two types of illudins known to exist. Illudins have a chemical structure entirely different from other chemotherapeutic agents. Illudin compounds were previously purified and submitted for evaluation to the National Cancer Institute Division of Cancer Treatment (NCI DCT) in vivo drug screening program but had a low therapeutic index in other experimental tumor systems in accordance with NCI studies. The extreme toxicity of illudins has prevented any applications in human tumor therapy.

Thus, there exists a need for chemotherapeutic agents which are toxic to tumors, and especially mdr tumors, and have an adequate therapeutic index to be effective for in vivo treatment. The subject invention satisfies this need and provides related advantages.

SUMMARY OF THE INVENTION

A method of inhibiting tumor cell growth in a subject is provided comprising contacting the tumor with a therapeutic amount of an illudin S or illudin M analog having the structure



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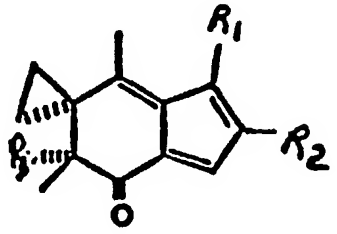


wherein the analog is capable of inhibiting tumor cell growth without excessive toxicity to the subject and wherein

R_1 is an alkyl or hydrogen;
R_2 is an alkyl; and
R_3 is an alcohol or ester.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the sensitivity of breast carcinoma and myeloid leukemia cells versus other tumors to illudin S.

Figure 2 shows the active sites of illudin S.

Figure 3 shows an NMR spectrum demonstrating the presence of a short acting intermediate in acid. Signal A is from the hydrogen on the double bond in the 5 membered ring (illudin M). Signal B is from the hydrogen atom on the short lived intermediate that results from the cyclopropane ring opening up (but before the double bond reacts). Signals marked at C are from the product that results when the double bond has reacted. With time, the signal peaks from illudin M will disappear and the peaks at position C will be the predominate signals. Signal B will disappear concurrently with Signal A confirming it is a short lived intermediate arising from illudin M.

Figure 4 shows the effect of illudin S on Molt-4 tumor growth in athymic mice (Balb/c).

Figure 5 shows the effect of dehydroilludin M on tumor growth.

Figure 6 shows the response of HL60/MRI xenograft to dehydroilludin M.

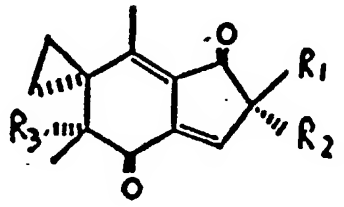
Figure 7 shows illudin S uptake using relatively sensitive HL60 cells and resistant B cells.

Figure 8 shows the rapid intracellular accumulation of illudin S by HL60 cells was saturated at high concentrations.

Figure 9 shows the analysis of the initial uptake of illudin S by HL60 cells at varying concentrations conformed to Michaelis-Menton saturation constants.

DETAILED DESCRIPTION OF THE INVENTION

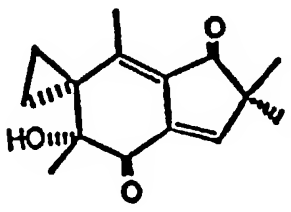
A method of inhibiting tumor cell growth in a subject is provided comprising contacting the tumor with a therapeutic amount of an illudin S or illudin M analog having the structure

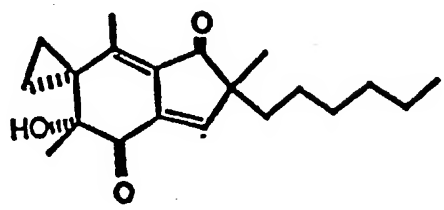


wherein the analog is capable of inhibiting tumor cell growth without excessive toxicity to the subject and wherein

R_1 is an alkyl or hydrogen;
R_2 is an alkyl; and
R_3 is an alcohol or ester.

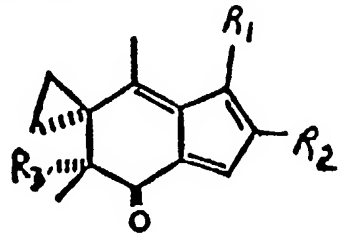
The analog can be any compound having the stated structure. Two examples of the effective analogs are:





A method of inhibiting tumor cell growth in a subject

is also provided comprising contacting the tumor with a therapeutic amount of an illudin S or illudin M analog having the structure



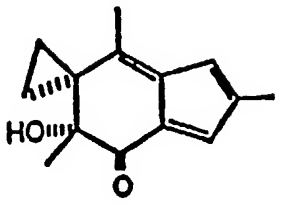
wherein the analog is capable of inhibiting tumor cell growth without excessive toxicity to the subject and wherein

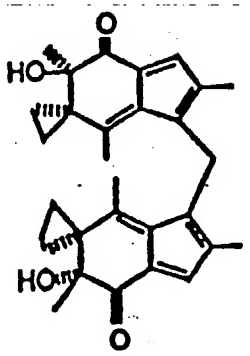
R_1 is an alkyl, alkoxyl or hydrogen;

R_2 is an alkyl; and

R_3 is an alcohol or ester.

The analog may be any compound having the stated structure. Two examples of effective anlogs are:





By "inhibiting" is meant either decreasing the tumor cell growth rate from the rate which would occur without treatment or causing the tumor cell mass to decrease in size. Inhibiting also includes a complete regression of the tumor. Thus, the analogs can either be cytostatic or cytotoxic to the tumor cells.

The subject can be any animal having a tumor. The analogs are effective on human tumors <u>in vivo</u> as well as on human tumor cell lines <u>in vitro</u>.

The tumor can be contacted with the analog by any effective means, many of which are well known in the art. The route of administration to the subject can include intravenous, oral, intraperitoneal, and oral and nasal inhalation. The preferred route of administration depends on the subject and type of tumor encountered.

Applicants have made the surprising discovery that analogs of illudin S and M can be made which are less toxic than illudin S and M but are a more effective chemotherapeutic agent in vivo. As noted above, illudin S and M have a low therapeutic index due to the extreme toxicity and, therefore, cannot be used therapeutically in humans. Applicants have discovered that various modifications in illudin S and M inhibit nucleophiles from reacting with the compound. This results in less facile opening of the cyclopropane ring and reduces the toxicity of the compound in vivo while still maintaining a high therapeutic index.

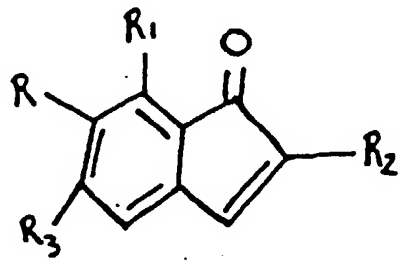
The various R groups recited define areas which do not affect the nucleophile reactivity of the analogs and, therefore, can be a wide variety of substituents. Thus applicants intend that the various R groups recited be construed broadly, for example, alkyl includes any structure which is attached to the alkyl group, i.e. an alkylfulvene.

The therapeutically effective amount of analog varies with the subject. However, it has been found that relatively high doses of the analogs can be administered due to the decreased toxicity over illudin S and M. A therapeutic amount between 30 to 1000 μg per kg of body weight has been found especially effective for intravenous administration while 300 to 60,000 or 1,200,000 μg per kg of body weight is effective if administered intraperitoneally. As one skilled in the art would

recognize, the amount can vary depending on the method of administration. Further, the amount can vary if the analog is linked to a toxin.

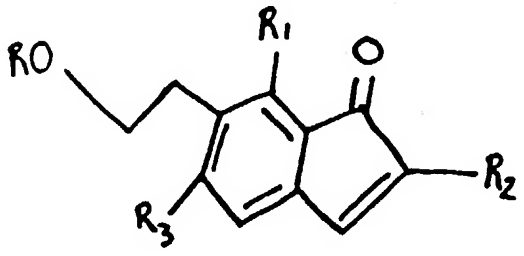
The analogs can be attached to a reagent to form a complex which binds to a tumor-associated antigen. Such methods are well known in the art and can include a linker which serves to connect the reagent to the analog. Such attachment can include any chemical bond, as for example a covalent bond. The reagent can be any reagent which specifically binds to a tumor-associated antigen on the tumor cell or in the tumor cell area. Typically such reagent is an antibody, either polyclonal or monoclonal. These complexes can then be used in therapy. The methods of the invention can be practiced on any tumor cells but are especially effective against tumor cells of myeloid, epidermoid, T-cell leukemia, and lung, ovarian and breast carcinoma.

Also disclosed is a compound having the structure



wherein R = methyl or hydroxyl; and R_1, R_2 and R_3 = methyl or alkyl.

Also disclosed is a compound having the structure



R = H or methyl; and R_1 R_2 and R_3 = methyl or alkyl.

EXAMPLE I

Synthesis of Dehydroilludin M

A mixture of illudin M (200 mg) and pyridinium dichromate (1 g) in dry dichloromethane (60 ml) was stirred at room temperature in a flask equipped with a rubber septum so that an atmosphere of argon could be maintained. After 20 hours, the reaction mixture was diluted with diethyl ether (20 ml) and filtered through a short column of silica gel. The column was further eluted with more diethyl ether and the combined filtrate was concentrated, giving a residue which was chromatographed on silica gel with hexane-ethyl acetate (10:1) as eluent. The desired compound was obtained in early fractions from the chromatography. The yield was 140 mg of white crystals melting at 64-65°C. NMR spectral data were recorded for this compound.

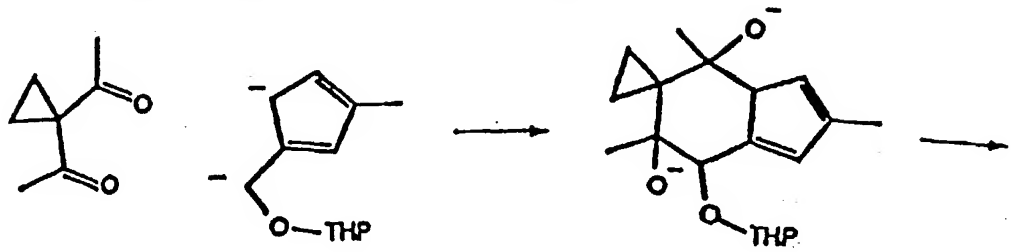
EXAMPLE II

Synthesis of Fulvene

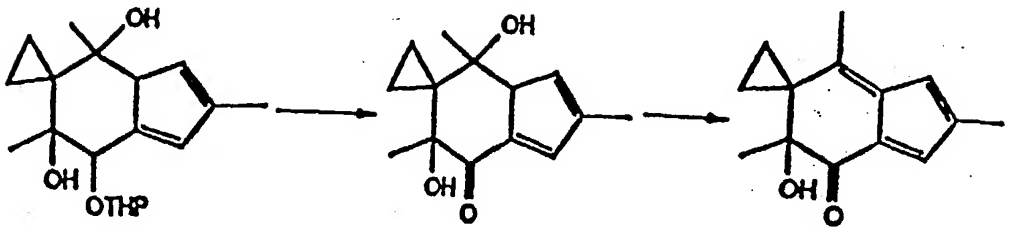
Illudin S (50 mg) was dissolved in water (2 mL) and 3N hydrochloric acid (2 mL) added to the solution. The resulting solution soon became cloudy (within 30 min) and a yellow precipitate formed. The mixture was placed in the refrigerator overnight; then it was extracted with chloroform (10 mL). The yellow chloroform solution was dried ($MgSO_4$) and the solvent was removed under reduced pressure leaving an orange-yellow gum. This material was chromatographed on silica gel with hexanes : ethyl acetate (6 :1) as eluent giving the fulvene (20 mg) and the bisfulvene (10 mg). NMR spectral data were recorded for

these compounds.

Alternatively, a total synthesis of the fulvene can also be achieved in the following way:



(THP = tetrahydropyranyl)



Reaction of the known 1, 1-diacetyl cyclopropane with the dianion of the cyclopentadiene derivative shown gives a diol which, on mild acid treatment followed by oxidation of the secondary hydroxyl, gives the diolketone. Selective elimination of a tertiary hydroxyl group gives the desired fulvene.

EXAMPLE III

In Vitro Studies

To assess cytotoxic effects, various concentrations of illudins were added to cultures of cells for 48 hours, then cell growth/viability was determined by trypan blue exclusion. As an alternative to 48 hour continuous exposure studies, cells were plated in liquid culture in 96 well plates, exposed to various concentrations of illudins for 2 hours, pulsed with [^{3}H]-thymidine for one to two hours and harvested onto glass filters. The filter papers were

added to vials containing scintillation fluid and residual radioactivity determined in a beta (scintillation) counter.

When screening the sensitivity of other solid tumor cell lines to illudin S, a breast cell line, MCF-7, was noted to be markedly sensitive (Figure 1). Another breast cell line maintained in our laboratory, MDA-231, was also found to be markedly sensitive to illudin S (Figure 1).

Studies with dehydroilludin M indicated this analog also displayed selective toxicity towards myeloid leukemia cells and breast carcinoma lines MCF-7 and MDA-231 (Table 1).

Table 1: Histiospecific cytotoxicity of illudin S and dehydroilludin M as demonstrated by inhibition of thymidine after a two hour exposure to the toxins (N - 3).

Compound	IC_{50} (nM/L) Illudin S	Dehydroilludin M
HL60, myeloid	7 ± 1	246 ± 19
8392, B-cell	236 ± 31	> 38,000
8402, T-cell	669 ± 196	> 38,000
242, melanoma	607 ± 70	> 38,000
547, ovarian	607 ± 110	> 38,000
SL-2, murine (thymic)	142 ± 15	5,235 ± 277
MCF-7, breast	58 ± 5	653 ± 65
MDA-231, breast	2.0 ± 0.2	112 ± 17

Because previous studies showed that CEM mdr variants were not resistant to illudin S, several other mdr cell types were studied for susceptibility to illudin S and the dehydroilludin M. These mdr daughter cell lines demonstrate a 200 to 800 fold increase in resistance to multiple conventional chemotherapeutic agents, but showed minimal or no resistance to illudin S or dehydroilludin M (Table 2). Thus, mdr cells associated with or without the gp170 protein were still susceptible to illudin toxicity. These studies indicate that illudins' novel structure

confers relative non-cross resistance in multidrug resistant hematopoietic cell lines. The derivative of illudins, dehydroilludin M, is slightly less potent than the parent illudin compound, but results (table 2) indicate that there is no cross-resistance to this compound in various mdr cell lines.

The effect of illudin S and dehydroilludin M on L1210, murine bone marrow CFU-gm, and C1498 (AML cell line) was studied. Illudin S was the most potent agent ever tested in this assay and displayed the largest differential effect ever noted between L1210 and AML leukemia lines and CFU-gm zone cites (Table 3). The derivative, dehydroilludin M, while less toxic was markedly more selective towards the AML line. It inhibited AML colony formation at concentrations where it had no effect on the CFU-gm cells (Table 4).

Table 2: Sensitivity of Different Mdr Lines to Illudin S

MDR cell line available		Illudin S	Dehydroilludin M
CEM Variants	Parent	8.3 ± 2.6	nt
	VM-1	16.2 ± 6.4	nt
	AraC	14	nt
	VLB100(gp170+)	3.7 ± 0.7	nt
	Dox (gp170+)	14	
MDA-231(Breast)	Parent	0.85 ± 0.23	54 ± 7
	3-1(gp170+)	0.89 ± 0.38	58 ± 11
MCF7-wt(Breast)	Parent	0.88 ± 0.11	92 ± 15
	ADR (GSH-transferase)	3.7 ± 0.4	68 ± 15
HL-60	Parent	3.1 ± 1.1	163 ± 11
	ADR (gp150+)	1.9 ± 0.8	191 ± 44
KB variant	Parent	0.58 ± 0.12	125 ± 14
	C-1 (gp170+)	0.69 ± 0.15	80 ± 18
	VBL(gp170+)	0.69 ± 0.11	78 ± 19
L1210	Parent	0.42 ± 0.08	62 ± 8
	DDPt(cis-plat)	0.46 ± 0.12	119 ± 39
	BCNU	0.58 ± 0.08	100 ± 31
	PAM(melphalan)	0.62 ± 0.15	73 ± 31
	CPA(cyclophos)	0.46 ± 0.12	38 ± 15

Table 3: Inhibition of Growth by Illudin S

Illudin S Concentration (ug/disc)	Zone of Inhibition		
	L1210	Go	Colon 38
2.50	500	240	30
1.25	400	70	0
0.63	320	30	0

Table 4: Effect of Illudins on Colony Formation

Compound	Dilution	Zone Size		
		L1210	CFU-GM	C1498 (AML)
Illudin S	1/1,000	850	400	> 1000
	1/4,000	600	200	800
	1/16,000	550	0	550
	1/64,000	300	0	250
Dehydroilludin M	1/25	400	200	> 1000
	1/125	200	100	750
	1/125 (repeat)	300	50	700
	1/625	100	0	400

EXAMPLE IV

Structure Function Studies

The structure-function studies were performed by synthesizing derivatives of the illudins and examining their *in vitro* toxicity for HL60 leukemia cells (Table 5). This study identified three critical sites for illudin toxicity. These include the cyclopropane ring (site A), the alpha/beta unsaturated bond site (site B), and the 7-ketone group (site C) (Figure 2). Alteration of any of these sites resulted in up to a 4 log decrease in toxicity. In contrast, the non-ring primary hydroxyl group (Figure 2,

site D) does not contribute to toxicity. Various large chemical groups can be attached to this site without altering toxicity. Many of the derivatives with a marked decrease in toxicity (as compared to illudin S or M) are still more potent than conventional chemotherapeutic agents such as BCNU or cis-platinum (Table 5).

Table 5: IC_{50} for Various Illudin Derivatives Versus Other Agents in HL-60 cells

COMPOUNDS	nM
Illudin S or M	10
Dihydroilludin S or M	100,000
Acylfulvene	500
Dehydroilludin M (diketone)	46
Isoilludin M	3,800
Ptaquiloside	7,700
Pterosin C	12,500
2,5,6,7-tetramethylindenone	475
Illudin tosylate	38
DNA polymerase inhibitor: Aphidocolin	2,100
Alkylating agent: BCNU	23,300
Crosslinking agent: cis-platinum	17,000
Alkylating agent: MNNG	15,000
Protein Synthesis Inhibitor: Ricin	0.2

EXAMPLE V

Structure-Function Studies: Chemical

Illudin M is readily converted to stable aromatic compounds (on treatment with dilute HCl) which in cell culture studies are more than 1,000 fold less toxic. The chlorine-carbon bond formation, cyclopropane ring opening and extrusion of the tertiary hydroxyl (as water) are synchronous. The intermediate formed can be detected by NMR spectroscopy of the reaction mixture (Figure 3). The intermediate, however, is highly reactive and is quickly converted to a phenol by attack of a second nucleophile,

i.e., water. Thus, under acidic conditions, illudin M is clearly bifunctional.

The above studies indicate that the toxicity of illudins is related to the ease with which the tertiary hydroxyl can be removed and the cyclopropane ring opened. It was found that illudin toxicity depends on the combined effects of the cyclopropane group (site A, Figure 2), the two double bonds (conjugated diene) (site B), and the 7-ketone (site C) towards electron resonance (or delocalization) in the illudin molecule. It was hypothesized that oxidation of the secondary 3'-hydroxyl group in the five membered ring to a ketone would alter the potency or selectivity of the molecule by contributing to further electron delocalization within the molecule. The new ketone group acts as an "electron sink" so that electrons of the cyclopropane C-C bonds are delocalized towards the ketone rather than to the carbon atom bearing the tertiary hydroxyl. This means the incipient carbocation, forming as the carbon-oxygen bond breaks, is not as stable as in the case of illudin M. Therefore, carbon-oxygen bond breaking is less favorable and reactivity is reduced. This ketone derivative, termed dehydroilludin M, was synthesized and was less toxic to HL-60 cells in vitro than illudin S or M (Table 4). As discussed above, the toxicity of dehydroilludin M appeared relatively selective for myeloid and breast carcinoma cells in vitro (Figure 1 and Table 1).

Consistent with the above hypothesis are the results of the kinetics of the reaction of illudin M and dehydroilludin M with dilute HCl. In dilute HCl, illudin M undergoes a pseudo first-order reaction ($k = 4.7 \times 10^{-3}$ min^{-1}, $t1/2 = 148$ minutes). Dehydroilludin M also demonstrated first-order kinetics but the reaction was considerably slower ($k = 2 \times 10^{-4}$ min^{1}, $t1/2 = 2765$ min). In the reaction with dehydroilludin M, no intermediate could be detected by

NMR spectroscopy. Presumably it formed too slowly and is too short-lived to be detected. The lower reactivity shown by dehydroilludin M suggests it is more selective in its reaction with nucleophiles and thus has a lower toxicity compared to illudin M.

The reaction of illudins with a naturally occurring nucleophile, glutathione has also been studied. At a wide pH range, from pH 3 to pH 9, glutathione spontaneously reacts with illudin M, illudin S, or dehydoilludin M, producing products analogous to that from the reaction of illudin M and HCl. The reaction rate is optimized at a pH of 6.1 to 7.0, indicating the reaction could occur intracellularly.

The toxicity of illudins towards a breast cell carcinoma line MCF7-wt and its MDR resistant daughter line MCF/Adr was then studied. The gp170 negative daughter cell line is drug resistant on the basis of a 50 fold increase in glutathione transferase, which results in a 200 to 800 fold decrease in sensitivity to conventional chemotherapeutic agents. This line also shows a 4.1 fold decrease in glutathione content. This daughter line showed a 4.2 fold decrease in sensitivity to illudin S (parent IC_{50} 0.88 nmoles/1; daughter line 3.70 nanomoles/1) versus the 200 to 800 fold seen with other agents. Kinetic studies on the ability of illudins to inhibit glutathione transferase indicated there was no direct inhibition of enzyme activity. These findings show that illudin toxicity is inversely correlated with intracellular glutathione content but not with glutathione transferase activity.

EXAMPLE VI

Animal Studies

Using procedures set forth in Leonard, J.E. et al., Cancer Res. 47:2899-02 (1987) and Dillman, R.O. et al, Cancer Res. 45:5632-36 (1985), both incorporated by reference herein, Molt-4 (human T-cell leukemia) xenografts were established in four week old athymic Balb/c nu/nu mice. After 3 weekly doses of total body radiation (600 cGy), mice were given subcutaneous flank injections of Molt-4 cells together with irradiated (6000 cGy) HT-1080 feeder cells. Two animals received only irradiated HT-1080 feeder cells to ensure these cells did not induce tumors. Animals were monitored for Molt-4 tumor development and when tumors were palpable (approximately 4 x 4 mm at 5 to 7 days), mice were randomized into groups of 5 as previously described. Control mice received intraperitoneal saline and treated mice received either 300 μg/kg illudin S, 30 μg/kg, or 30 μg/kg dehydroilludin M, IP twice weekly. In mice given illudin S there was tumor growth delay (Figure 4).

In contrast, in nude mice which received the dehydroilludin M at the low dosage of 30 μg/kg (the compound was subsequently found to be nontoxic to mice at 1000 μg/kg IP twice a week), three of five tumors underwent complete regression, but two tumors failed to respond (figure 5). The two apparently resistant tumors were harvested and tested in vitro for resistance to illudin S and dehydroilludin M. There was no evidence of resistance to either compound. Two of the complete responders were followed for over twelve weeks without evidence of tumor regression.

Using a different source of athymic nude mice, these experiments were repeated. In these studies there was

little effect of illudins on tumor growth. The reason for this variability in response to Molt-4 xenografts probably relates to the low doses of dehydroilludin M, interanimal variations in glutathione metabolism, or drug distribution.

The efficacy of dehydroilludin M was then screened in a syngeneic model using murine SL-2 cells. SL-2 leukemia/lymphoma cells are injected subcutaneously and metastasized to lymph nodes, spleen, and lungs, and drug efficacy in this model is determined by increased life span (ILS). The SL-2 cells were administered at 2.5 million cells per animal and treatment was delayed for 7 days until the tumors were palpable. This is a relatively stringent test against established tumors and contrasts to general drug screens in the SL-2 model which normally use only 0.5 million cells and starting drug treatment at 3 days. Dehydroilludin M had a little effect at 30 mg/kg IP twice a week, ILS 5%, and 60 mg/kg IP twice a week, ILS 18%. When administered IV at 0.03 mg/kg, twice a week, the ILS increased to 38%. This suggests the drug is metabolized by the liver and is likely more efficacious when administered IV.

During the course of these in vivo experiments, it became clear from in vitro experiments, that histiospecificity of illudins depends upon the presence of an active energy-dependent pump. The SL-2 and the Molt-4 cells were studied and it was determined that the uptake mechanism was not present. Therefore, the studies were redirected into xenograft models that used cells of myeloid lineage.

Human HL-60 cells capable of growing as xenografts in nude mice without animal radiation were obtained from Dr. Theodore Brightman (NCI). These cells termed HL-60 MRI cells, were confirmed to have energy-dependent uptake pump, a not unexpected finding as their parental cells posses the

pump. Dehydroilludin M induced dose related tumor inhibition when administered IP on a twice a week schedule (figure 6). The MTD IP dose for dehydroilludin M was reached in these studies on the 2 dosages per week IP dose schedule. Similar tumor regressions have been observed with IV dehydroilludin M.

In collaboration, the in vivo effects of dehydroilludin M was again studied. Initially the compound was studied against L1210 cells. A dose of 2.5 mg/kg IP given daily for 5 days resulted in an ILS of only 9%. The dehydroilludin M was then administered as a 24 hour infusion (5.0 mg/kg); the ILS was 11%. After we became aware of the presence of the energy-dependent uptake in human myelocytic cells, dehydroilludin M was screened for in vivo efficacy against a syngeneic mouse AML model using C1498 cells and a single bolus of illudin S, 2.5 mg/kg IP, produced an ILS of 35%. A second trial using the same dosage, administered IP once a day for 5 days resulted in a 44% ILS. As the animals can tolerate 30 mg/kg IP or 1 mg/kg IV (tail vein) on a twice a week schedule for 4 weeks without demonstrating weight loss or a decrease in food/water intake, it is possible to optimize both dosage and treatment schedule.

EXAMPLE VII

HL60/MRI Mouse Experiment With Acylfulvene and Dehydroilludin M

Thirty mice were injected subcutaneously, over the shoulder, with 500,000 HL60/MRI cells (human myeloid leukemia tumor cells). Treatment was begun on day 11, rather than immediately. This delay in starting treatment is a stringent test to determine whether a compound is effective. By delaying treatment, the tumor cells become firmly established.

The mice were divided into 6 groups of 5 each. One group was the control and these animals received on a placebo, the solution used to dilute the agent. The other groups received the following compounds and dosages: the dehydroilludin M compound at 1.0 mg/kg, the dehydroilludin M at 3.0 mg/kg, the Acylfulvene at 0.3 mg/kg, the Acylfulvene at 1.0 mg/kg, the Acylfulvene at 3.0 mg/kg. All animals received the placebo or drugs by intravenous injection using a tail vein. The placebo or drugs were administered on a twice a week schedule.

Results are summarized in the accompanying table 6. Both the dehydroilludin M and the Acylfulvene compound were effective at inhibiting tumor growth and demonstrated dosage dependence inhibition (the more drug administered, the less the tumors grew). The animals receiving the highest amount of either drug did not display any evidence of adverse effect, such as a decrease in food or water intake, nor a statistically significant decrease in body weight. These results show that higher dosages of either drug can be administered. Also, that the drug could be administered on a more effective dosage schedule, such as on a daily basis.

TABLE 6

Summary: HL60/MRI experiment, intravenous - # 1

BY TOTAL TUMOR WEIGHT [Mg]

	DAY 11	DAY 18	DAY 25	DAY 32	DAY 40
CONTROL					
No Drug	99±36	845±282	3299±1080	10162±4123	16747±5061
Dehydroilludin M					
1 mg/kg IV	114±55	883±311	2274±992	6025±1772	11507±3707
3 mg/kg IV	101±40	911±309	2127±1092	2854±1260	4784±2303
Acylfulvene					
0.3 mg/kg IV	73±38	540±167	1352±520	3204±1147	9501±4605
1 mg/kg	58±32	582±297	964±685	2321±1434	6275±2865
3 mg/kg	38±30	369±250	336±215	437±238	1201±501

EXAMPLE VIII

General In Vitro Screening Procedures and Cell Uptake Studies

In keeping with the suggestions of the previous examples and our concentration on mechanisms of illudin action and tissue specificity, other myeloid leukemia cell lines can be screened for rapid illudin uptake (KG1, KG1a, HEL, K562, OCI-M1, AML-193).

The procedures for *in vitro* screening of illudin compounds are detailed in the previous examples. Cytotoxicity of new analogs for cell lines is initially evaluated over a 5 log range using growth or semi-solid colony forming assays, and inhibition of thymidine

incorporation. Inhibition of thymidine incorporation is used because earlier studies indicate that thymidine incorporation is preferentially inhibited by illudins and correlates closely with cell death. Analogs are screened against normal bone marrow progenitors and a variety of cell lines involving various leukemias, B and T cell) and solid tumors (melanoma, ovarian).

In vitro testing of dehydroilludin M on various cell lines, including MDR lines, can also be performed on DNA-repair deficient cell lines and normal bone marrow progenitors. A variety of other analogs can be prepared. Since these analogs will have alterations in the known active sites, they are expected to result in a similar tumor inhibition. Screening studies for these analogs can include various mdr cells (to ensure that no cross-resistance occurs) and DNA-repair deficient cell lines.

In vitro testing can also study sensitivity of other breast cell lines to determine if they are also preferentially sensitive to illudin S, dehydroilludin M, and the fulvene analog.

EXAMPLE IX

Assessment of Illudin Uptake in Tumor Cells

While human myeloid tumor cells are sensitive to illudins, their normal precursors, granulocyte/macrophage forming units, are relatively resistant to illudins by 1.5 to 2.0 logs, demonstrating that the transport system is absent from some normal marrow cells and providing a therapeutic margin of safety.

Specific illudin S uptake was assayed using relatively sensitive HL60 cells and resistant B cells. At 37°C, HL60 myeloid leukemia cells demonstrated rapid uptake of illudin

S, while the relatively insensitive 8392 B-cells exhibited comparatively little drug incorporation (Figure 7). The intracellular accumulation of illudins in the B cell line was slow and linear for 7 hours (r - 0.984), at which time the intracellular concentration approached that of the incubation mixture. HL60 cells, in contrast, rapidly accumulated the toxin and intracellular accumulation reach a plateau within one hour. HL60 cells exposed to 10 nM illudin S concentrated the toxin 19 fold, whereas B cells did not actively concentrate the toxin. The rapid intracellular accumulation of illudin S by HL60 cells was saturated at high concentrations (Figure 8). In contrast, illudin S accumulation in 8392 B cells remained concentration dependent. Analysis of the initial uptake of illudin S by HL60 cells at varying concentrations revealed that the influx of illudin S conformed to Michaelis-Menton saturation kinetics (Figure 9). The Vmax for HL60 cells was 27 picomoles/minute/mg of protein and the Km was 4.2 μM. This indicates HL60 cells have a very high transport capacity for illudins as the Vmax for illudins is 5 times the Vmax for folate, a vitamin required by cells.

Cold (4°C), 1% azide, and the metabolic blockers 2-deoxyglucose and antimycin A, all block uptake of illudin S into HL60 cells but have little effect on the insensitive 8392 B-cells (Table 7). These studies indicate that illudin S is transported and concentrated into HL60 cells by an energy dependent transport system, whereas the transport into insensitive B-cells occurs only by diffusion (passive or nonenergy requiring transport). MCF7 breast tumor cells also demonstrated inhibition of uptake by cold. The finding of an energy-dependent transport mechanism explains why myeloid and breast tumor cells are so sensitive to illudins with short exposure times, but B-cells are not.

TABLE 7

Uptake of [^{3}H] Illudin S by HL60 Myeloid versus 8392 B-cells

Maximum uptake per hour (picomoles)[a]

Conditions	HL60	8392	MCF7
37°C	75 ± 16[b]	5.5 ± 1.4	29 ± 4
4°C	4.3 ± 0.9	3.4 ± 1.0	4.0 ± 2.1
1% Azide	8.7 ± 1.4	4.3 ± 1.3	NT[b]
2-deoxyglucose & Antimycin A	16.7 ± 3.5	3.6 ± 1.4	NT

Cells were exposed to 100 ng/ml of [^{3}H]-labeled illudin S for one hour and harvested as described. Results are expressed as mean ± SE and represent 3 experiments.

[a]per 10 million cells

[b]NT = not tested

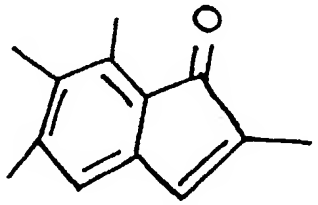
EXAMPLE X

Synthesis and Structure of 2,5,6,7-Tetramethyl-1-Indenone and Dehydropterosin Compounds

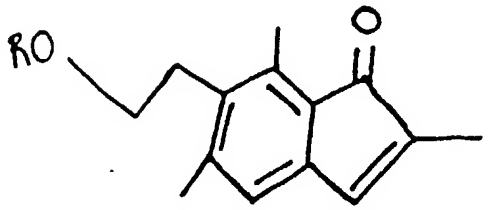
First 2,4,6-trimethyl-1,3-indanione was synthesized by preparing a solution of 1,2,3-trimethylbenzene and methylmalonylchloride in carbon disulfide and adding aluminum trichloride dropwise over two hours. The mixture was relfuxed for 2 more hours, crushed ice added, and extracted three times with chloroform. The combined extract was washed with brine, dried, and solvent removed to leave a residue which was purified by chromatography with 1% ethyl acetate in benzene. Removal of solvent and purification by sublimation gave the desired product.

The 2,5,6,7-tetramethyl-1-indenone was prepared by

reducing 2,4,5,6-tetramethyl-1,3-indanione with zinc dust at 50°C. Product was purified by chromatography with 1% ethyl acetate in benzene to yield two isomers. The major isomer was treated with 10% potassium hydroxide, then purified by sublimation. The compound has the structure:



Dehydropterosin O synthesis: 3-acetoxy-6(beta-methoxy) ethyl-2,5,7-trimethyl-1-indanone was dissolved in tetrahydrofuran and 10% potassium hydroxide and refluxed for two hours. The solution was then extracted three times with ether and the combined extracts chromatographed with 2% ethylacetate in benzene to yield the Dehydropterosin O compound. The compound has the structure:



R = H Dehydropterosin O

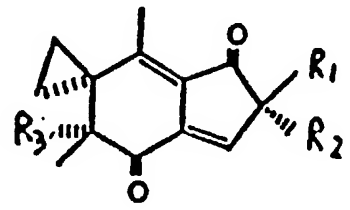
R = CH_3 Dehydropterosin B

Both compounds were toxic to cells in vitro and have antifungal properties.

Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A method of inhibiting tumor cell growth in a subject comprising contacting the tumor with a therapeutic amount of an illudin S or illudin M analog having the structure



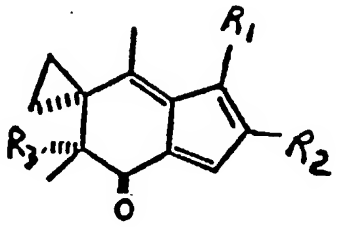
wherein the analog is capable of inhibiting tumor growth without excessive toxicity to the subject and wherein

R_1 is an alkyl, alkoxyl or hydrogen;

R_2 is an alkyl; and

R_3 is an alcohol or ester.

2. A method of inhibiting tumor cell growth in a subject comprising contacting the tumor cells with a therapeutic amount of an illudin S or illudin M analog having the structure



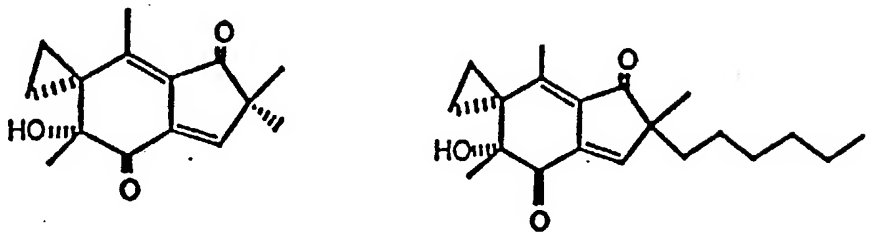
wherein the analog is capable of inhibiting tumor growth without excessive toxicity to the subject and wherein

R_1 is an alkyl or hydrogen;

R_2 is an alkyl; and

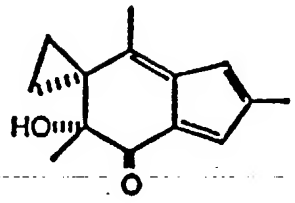
R_3 is an alcohol or ester.

3. The method of any of claim 1 wherein the compound is selected from the group consisting of:

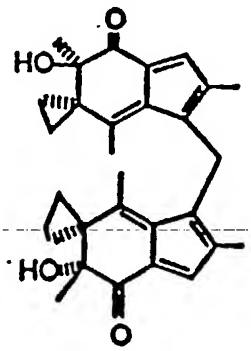


Dehydroilludin M

4. The method of claim 2 wherein the compound is selected from the group consisting of:



Fulvene



Bisfulvene

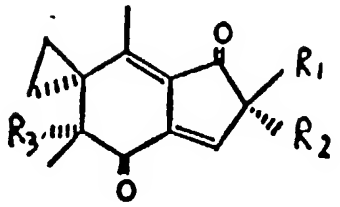
5. The method of claim 1 wherein the therapeutic amount is between 30 to 1,200,000 μg per kg of body weight.

6. The method of claim 2 wherein the therapeutic amount is between 30 to 60,000 μg per kg of body weight.

7. The method of claim 1 wherein the analog is administered by any means selected from intravenous, oral, intraperitoneal, and inhalation.

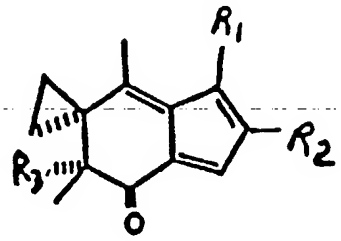
8. The method of claim 2 wherein the analog is administered by any means selected from intravenous, oral, intraperitoneal, and inhalation.

9. An illudin S or illudin M analog having the structure



wherein the compound is attached to a reagent to form a complex which binds to a tumor antigen.

10. An illudin S or illudin M analog having the structure



wherein the analog is attached to a reagent to form a complex which binds to a tumor antigen.

11. The complex of claim 9 wherein the reagent is an antibody.

12. The complex of claim 10 wherein the reagent is an antibody.

13. A method of inhibiting tumor cell growth in a subject comprising contacting the tumor with the complex of claim 11.

14. A method of inhibiting tumor cell growth in a subject comprising contacting the tumor with the complex of claim 12.

15. A therapeutic composition comprising 30 to 100 mg of the compound of claim 1 and a pharmaceutically acceptable carrier.

16. A therapeutic composition comprising 30 to 100 mg of the compound of claim 2 and a pharmaceutically acceptable carrier.

17. The method of 1, wherein the tumor cell is selected from the group consisting of myeloid, epidermoid, T-cell leukemia, and lung, ovarian and breast carcinoma.

18. The method of 2, wherein the tumor cell is selected from the group consisting of myeloid, epidermoid, T-cell leukemia, and breast carcinoma.

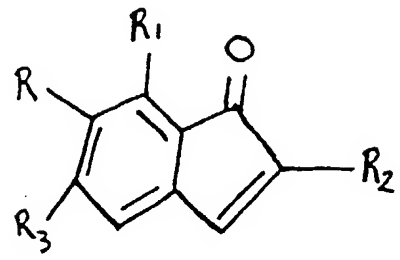
19. A method of synthesizing fulvene comprising:

(1) reacting a 1, 1-diacetyl cyclopropane with a dianion of a cyclopentadiene derivative to give a diol;

(2) treating the diol with acid and oxidizing the secondary hydroxyl to give a diolketone; and

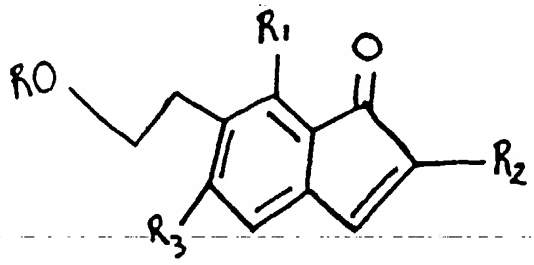
(3) selectively eliminating the tertiary hydroxyl group to give fulvene.

20. A compound having the structure



wherein R = methyl or hydroxyl; and R_1, R_2 and R_3 = methyl or alkyl.

21. A compound having the structure



R = H or methyl; and R_1 R_2 and R_3 = methyl or alkyl.

FIG. 1

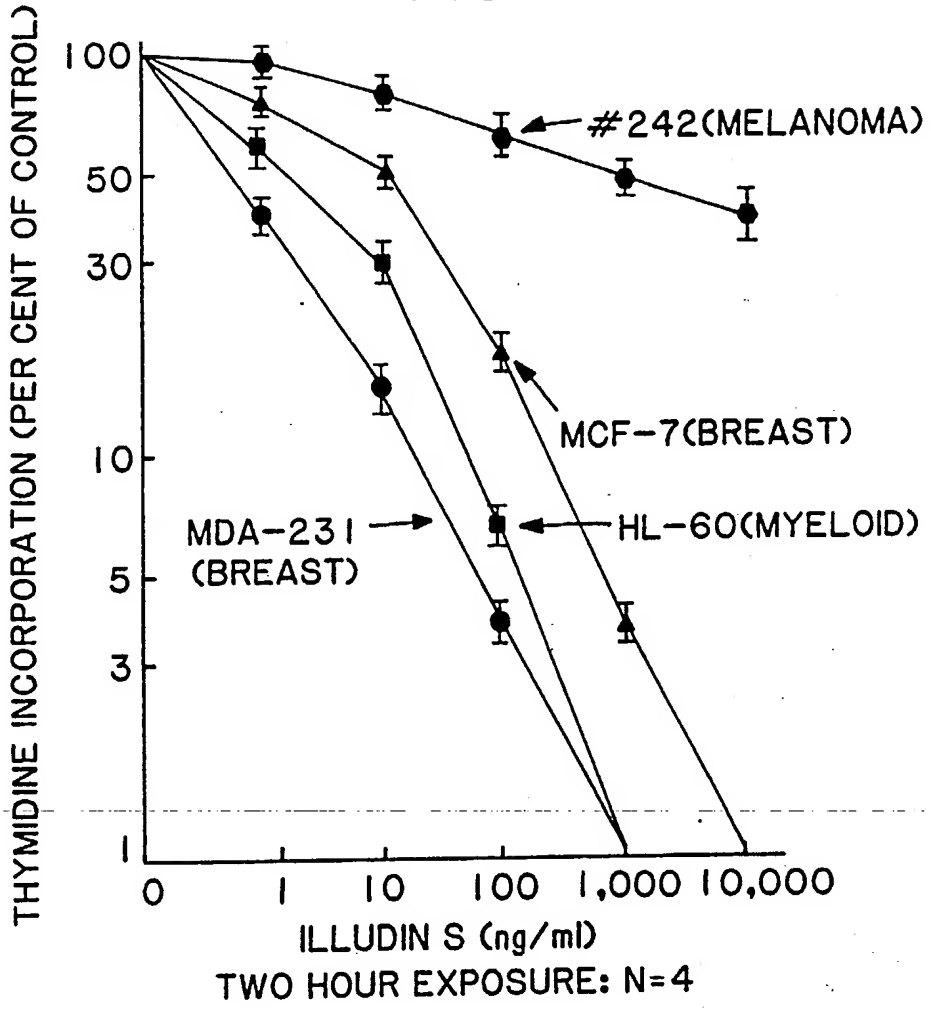
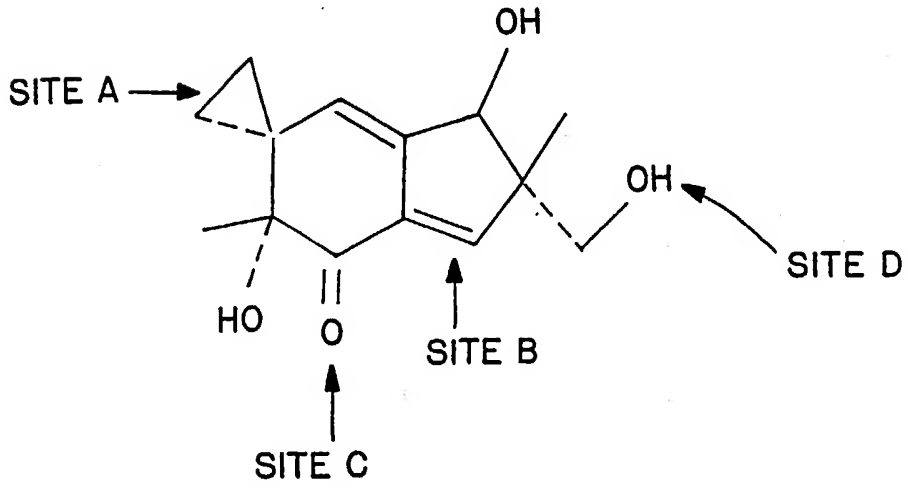

THYMIDINE INCORPORATION (PER CENT OF CONTROL)
100
50
30
10
5
3
1
0
1
10
100
1,000
10,000
ILLUDIN S (ng/ml)
TWO HOUR EXPOSURE: N=4
#242(MELANOMA)
MCF-7(BREAST)
HL-60(MYELOID)
MDA-231
(BREAST)

FIG. 2


SITE A
OH
OH
SITE D
HO
O
SITE B
SITE C

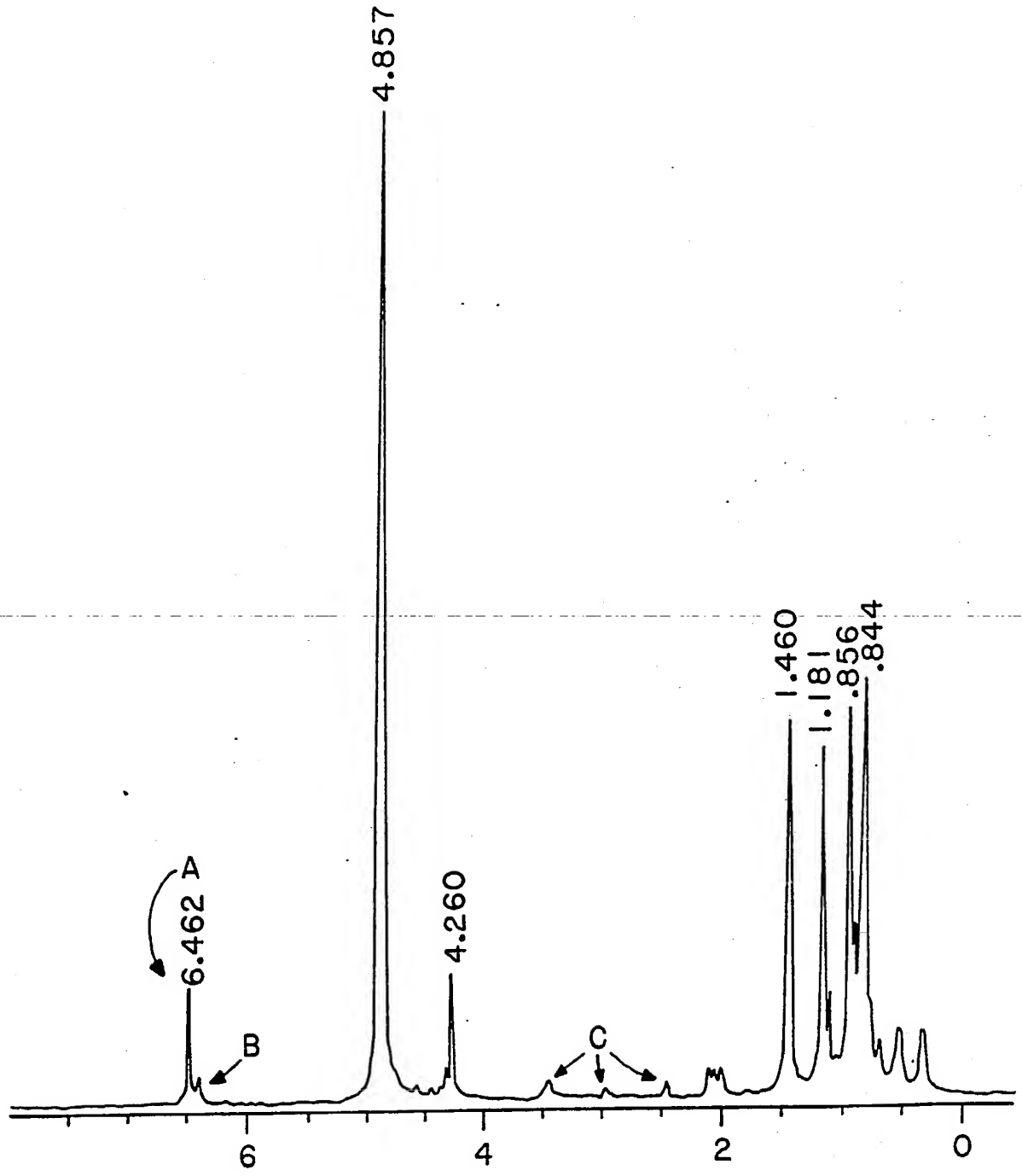


FIG. 3

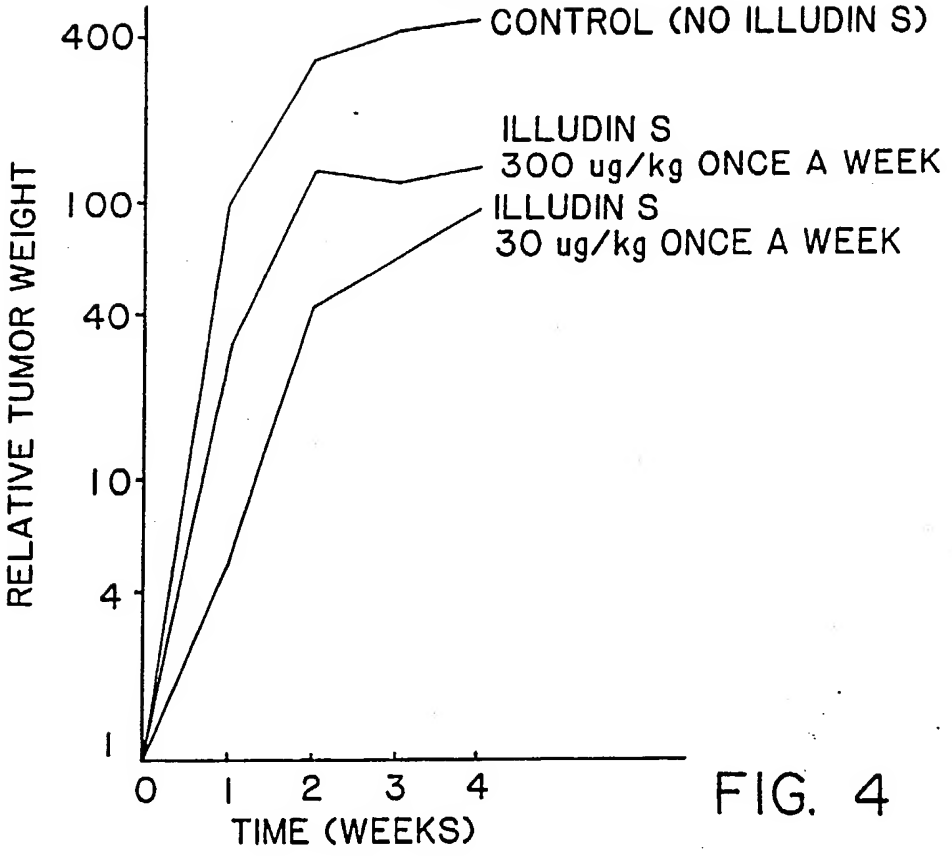

CONTROL (NO ILLUDIN S)
ILLUDIN S
300 ug/kg ONCE A WEEK
ILLUDIN S
30 ug/kg ONCE A WEEK
RELATIVE TUMOR WEIGHT
400
100
40
10
4
1
0
1
2
3
4
TIME (WEEKS)

FIG. 4

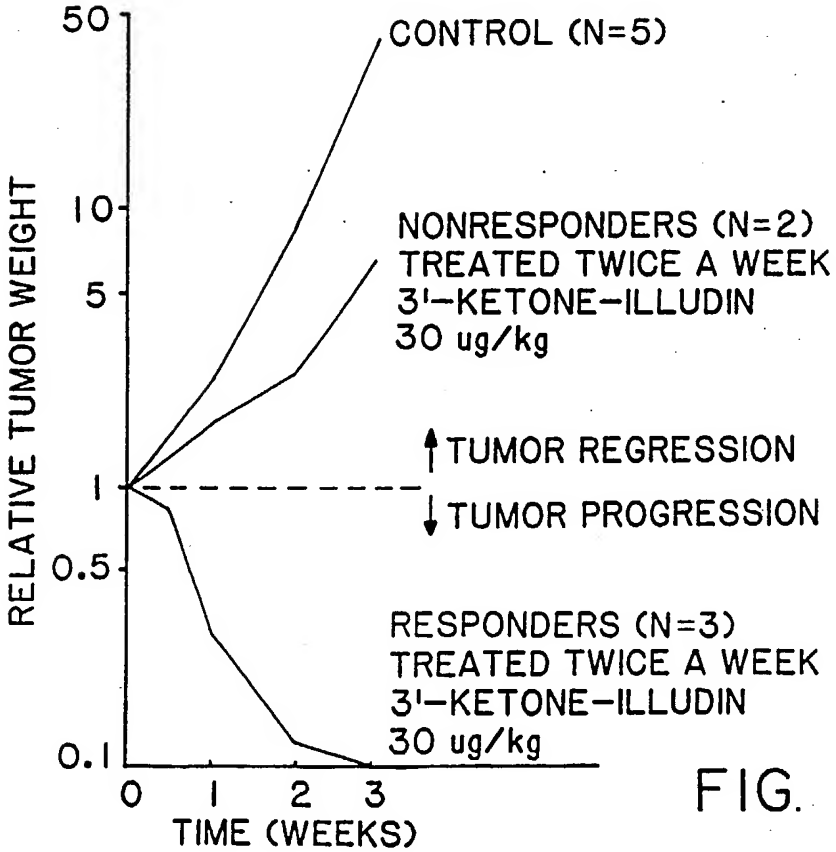

CONTROL (N=5)
NONRESPONDERS (N=2)
TREATED TWICE A WEEK
3'-KETONE-ILLUDIN
30 ug/kg
TUMOR REGRESSION
TUMOR PROGRESSION
RESPONDERS (N=3)
TREATED TWICE A WEEK
3'-KETONE-ILLUDIN
30 ug/kg
RELATIVE TUMOR WEIGHT
50
10
5
1
0.5
0.1
0
1
2
3
TIME (WEEKS)

FIG. 5

FIG. 6

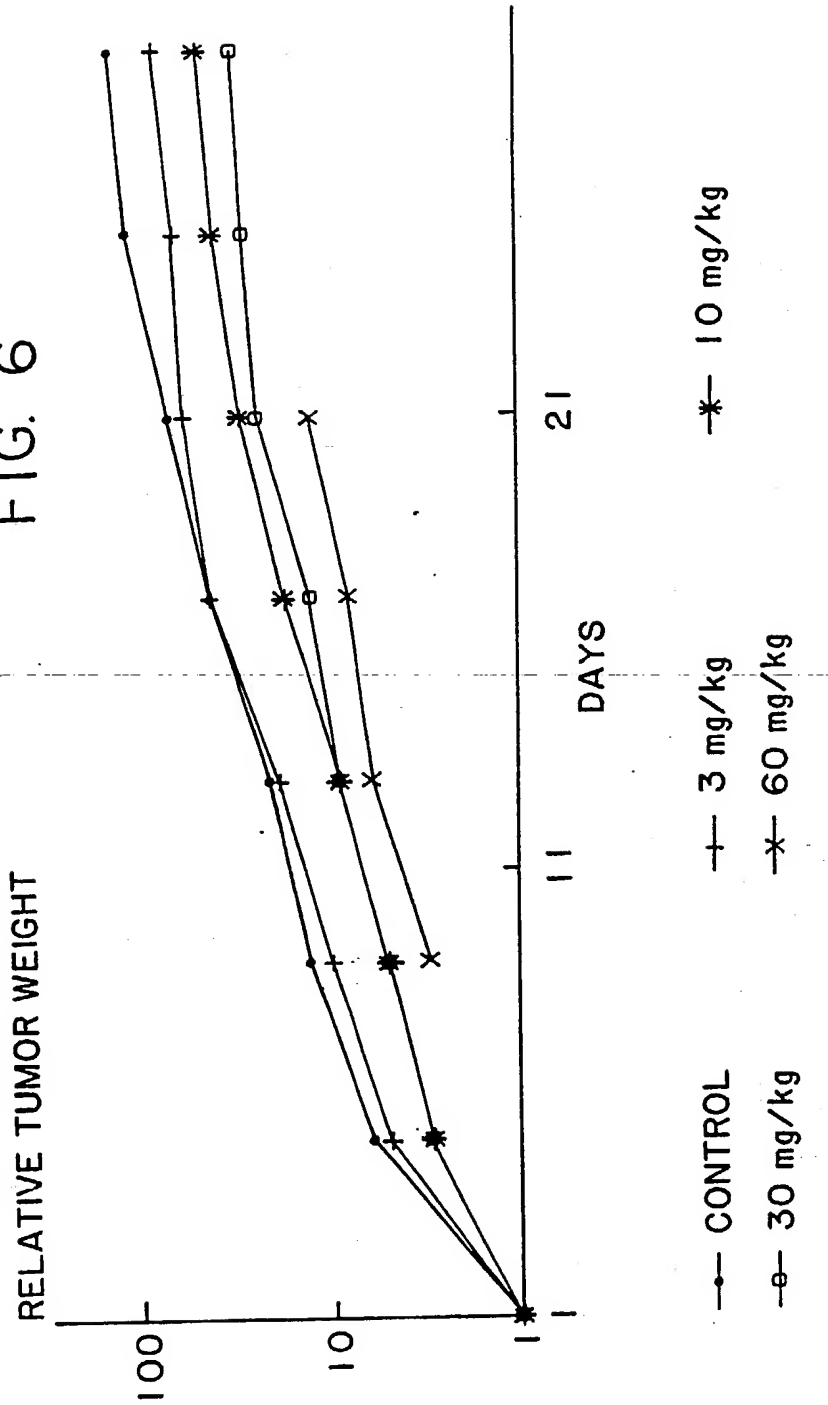

RELATIVE TUMOR WEIGHT
100
10
1
1
11
21
DAYS
CONTROL
3 mg/kg
10 mg/kg
30 mg/kg
60 mg/kg

FIG. 7

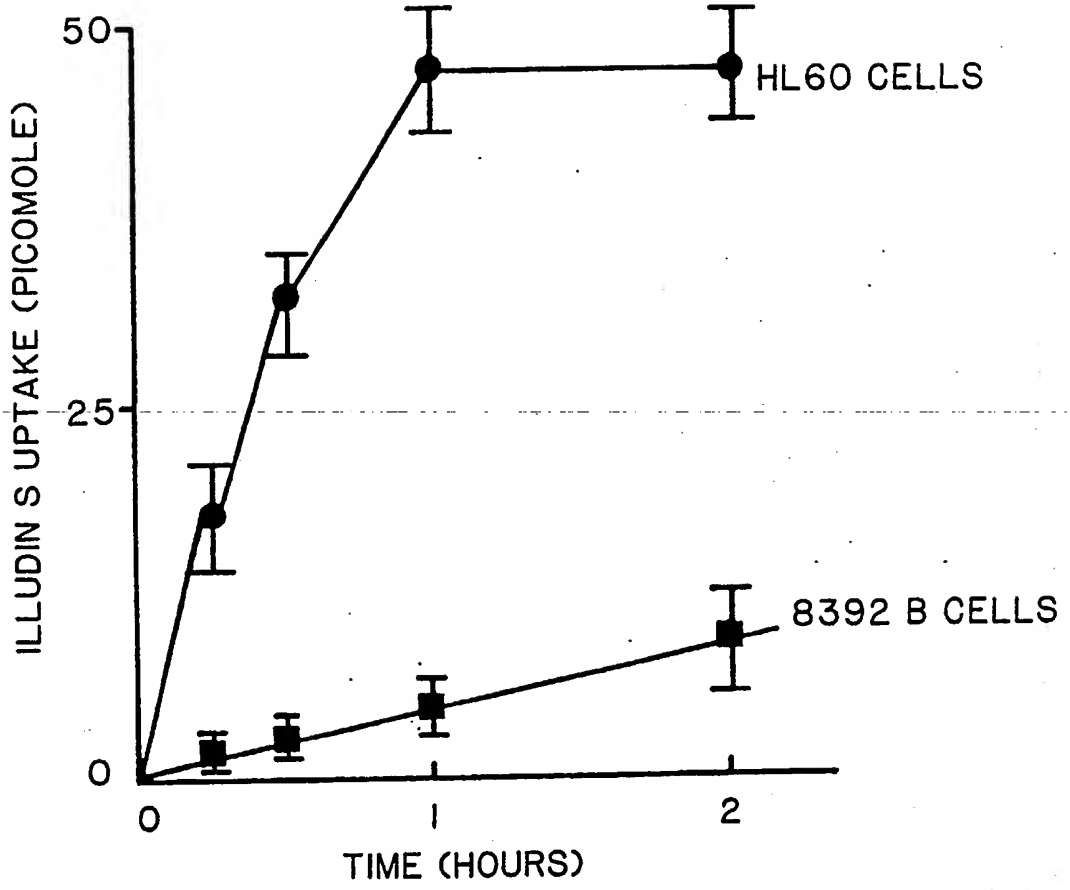

50
25
0
ILLUDIN S UPTAKE (PICOMOLE)
HL60 CELLS
8392 B CELLS
0
1
2
TIME (HOURS)

FIG. 8

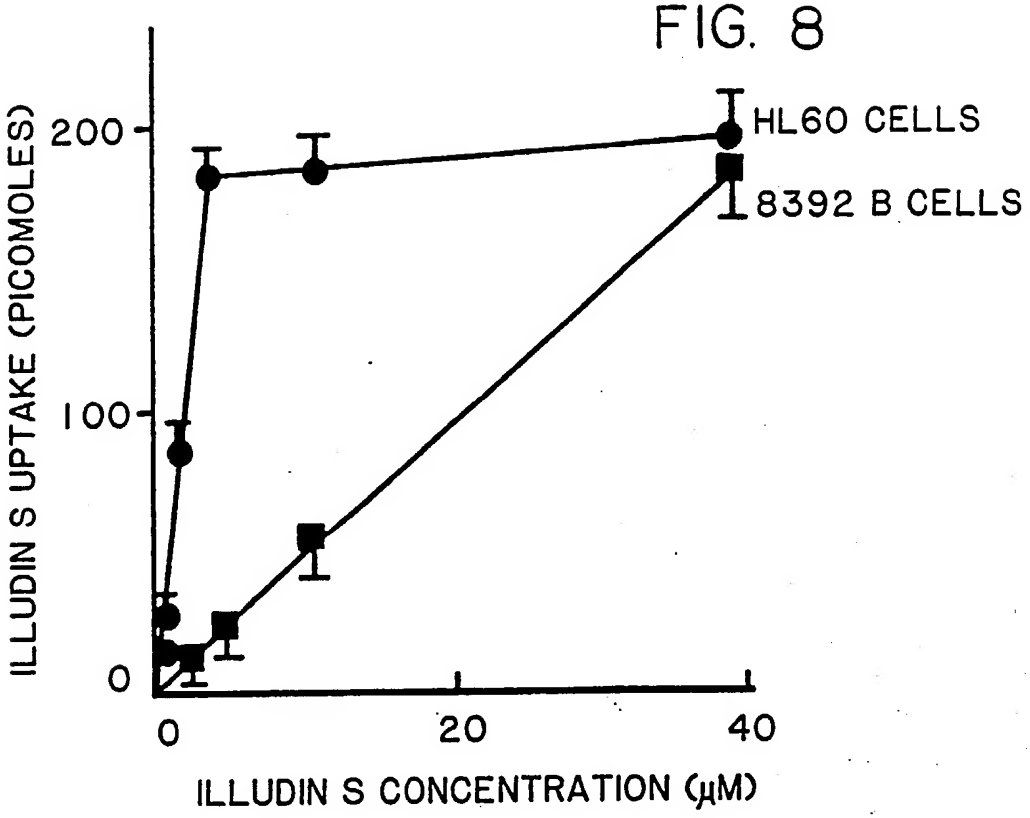
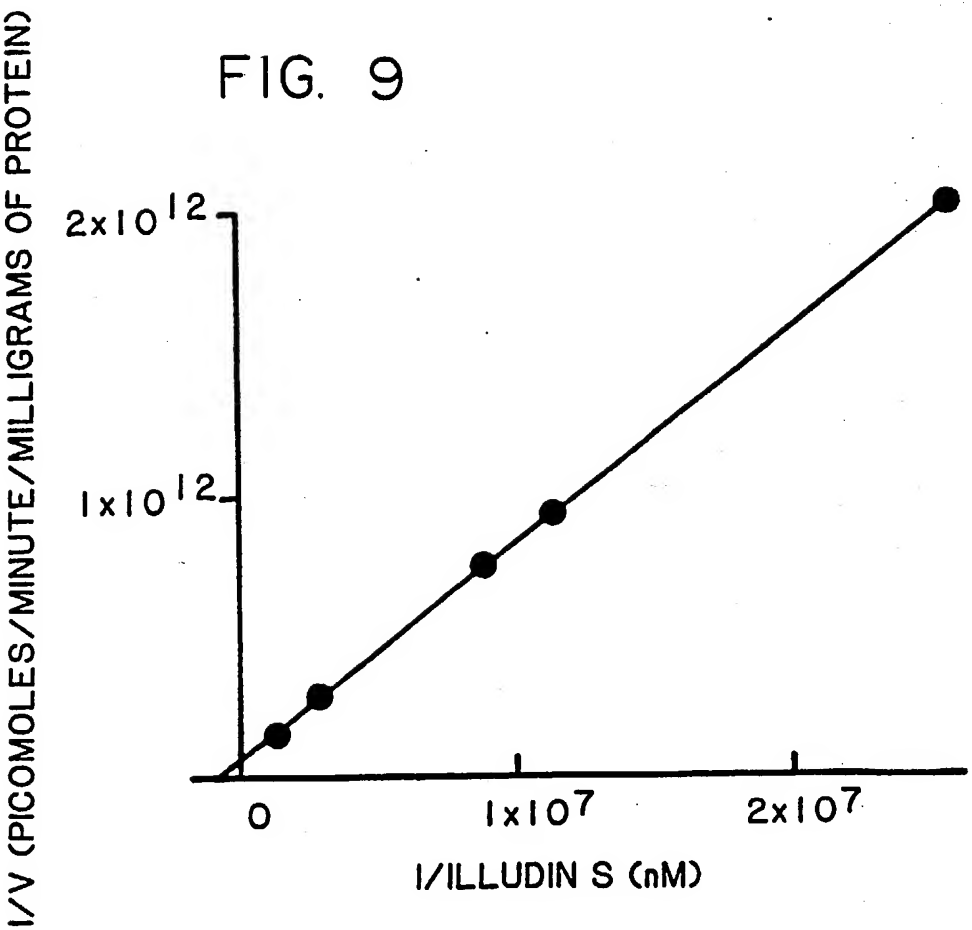

HL60 CELLS
8392 B CELLS
ILLUDIN S UPTAKE (PICOMOLES)
200
100
0
0
20
40
ILLUDIN S CONCENTRATION (μM)

FIG. 9


I/V (PICOMOLES/MINUTE/MILLIGRAMS OF PROTEIN)
2x10 12
1x10 12
0
1x10 7
2x10 7
I/ILLUDIN S (nM)